The role of protein kinase C in the inactivation of hepatic glycogen synthase by calcium-mobilizing agonists

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The regulation of glycogen synthase by Ca^{2+} -mobilizing hormones was studied by using rat liver parenchymal cells in primary culture. Long-term exposure of hepatocytes to 4 β -phorbol 12-myristate 13-acetate (TPA) resulted in a decrease in vasopressin or ATP inhibition of glycogen synthesis and glycogen synthase activity, without any change in the activation of glycogen phosphorylase. In contrast, treatment with TPA did not diminish the effects of glucagon, isoprenaline or A23187 on glycogen synthase or phosphorylase. TPA treatment for 18 h did not change specific [^3H]vasopressin binding, but abolished protein kinase C activity in a concentration-dependent manner. The effects of TPA to decrease protein kinase C activity and to reverse the inactivation of glycogen synthase by vasopressin were well correlated and were mimicked by mezerein, but not by 4 α -phorbol. However, 1 μM -TPA totally inhibited protein kinase C activity, but reversed only 60 % of the vasopressin effect on glycogen synthase. It is therefore concluded that Ca^{2+} -mobilizing hormones inhibit glycogen synthase partly, but not wholly, through a mechanism involving protein kinase C.

INTRODUCTION

It is well acknowledged that a large variety of extracellular messengers, including vasopressin, angiotensin II and ATP, elicit their responses by inducing the breakdown of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate in their target cells (Berridge, 1984; Berridge & Irvine, 1984). Inositol 1,4,5-trisphosphate then induces intracellular Ca^{2+} mobilization from a non-mitochondrial pool. In addition, there is accumulation of 1,2-diacylglycerol, which is formed from phosphatidylinositol 4,5-bisphosphate (Berridge, 1984; Berridge & Irvine, 1984) and probably other phospholipids (Bocckino *et al.*, 1985). Diacylglycerol also acts as an intracellular second messenger, because it activates protein kinase C (Nishizuka, 1984).

In the liver, glycogen metabolism is mainly controlled by two enzymes: glycogen synthase and glycogen phosphorylase. Ca^{2+} -mobilizing hormones inactivate glycogen synthase and activate glycogen phosphorylase by phosphorylation mechanisms. Glycogen synthase can be phosphorylated *in vitro* at multiple sites by different Ca^{2+} -dependent protein kinases, including phosphorylase *b* kinase, Ca^{2+} /calmodulin-dependent protein kinase and protein kinase C (Roach *et al.*, 1978; Payne & Soderling, 1980; Ahmad *et al.*, 1982, 1984; Payne *et al.*, 1983; Imazu *et al.*, 1984; Camici *et al.*, 1984; Akatsuka *et al.*, 1984; Arino & Guinovart, 1986). However, it is still unclear how vasopressin, angiotensin II and ATP cause inactivation of glycogen synthase.

Roach & Goldman (1983) first showed that 4 β -phorbol 12-myristate 13-acetate (TPA), a tumour-promoting phorbol ester, inhibited glycogen synthase in hepatocytes without modifying the activity of glycogen phosphorylase. Phorbol esters are believed to act by substituting

for diacylglycerol in the activation of protein kinase C (Castagna *et al.*, 1982), although other actions are possible. Ahmad *et al.* (1984), using protein kinase C from brain, showed phosphorylation of glycogen synthase from rabbit muscle and liver. For the muscle enzyme, phosphorylation was shown to be associated with inactivation. On the other hand, Imazu *et al.* (1984) and Nakabayashi *et al.* (1987) showed that protein kinase C phosphorylated liver glycogen synthase, but was ineffective in causing inactivation.

Because of these contradictory reports we have undertaken, using primary cultures of rat hepatocytes, a study to define the role of protein kinase C in the regulation of hepatic glycogen synthase by certain agonists. Although several protein kinase C antagonists have been described (Kawamoto & Hidaka, 1984; Shoji *et al.*, 1985), we have not employed these, because of their poor potency and because they block other protein kinases (see DeRiemer *et al.*, 1985). We have therefore utilized protein kinase C-deficient cells obtained by chronic treatment with tumour-promoting phorbol esters. This approach has been employed for Swiss 3T3-L1 (Blackshear *et al.*, 1985), GH $_3$ rat pituitary-tumour cells (Ballester & Rosen, 1985), foetal-rat skin keratinocytes (Chida *et al.*, 1986) and MCF-7 human breast-cancer cells (Fabbro *et al.*, 1986).

EXPERIMENTAL

Isolation and primary culture of hepatocytes

Male Sprague-Dawley rats (200–250 g) fed *ad libitum* were used. Hepatocytes isolated by perfusion of livers with collagenase (Blackmore & Extton, 1985) were washed three times with Dulbecco's modified Eagle

Abbreviations used: TPA, 4 β -phorbol 12-myristate 13-acetate; AVP, [8-arginine]vasopressin; DTT, dithiothreitol; DMEM, Dulbecco's modified Eagle medium.

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medium (DMEM) supplemented with 1% (w/v) bovine serum albumin, and then filtered through a sterile nylon stocking and sedimented by centrifugation at 50 *g* for 30 s. The cells were finally suspended in DMEM supplemented with bovine serum albumin (1%), dexamethasone (1 μ M), defined foetal bovine serum (10%, v/v), penicillin (100 units/ml) and streptomycin (0.1 mg/ml). Primary cultures were started by seeding viable (90–95%) cells on to plastic tissue-culture dishes or culture-well plates (3×10^4 – 4×10^4 cells/cm²).

After plating for 4 h at 37 °C under a humidified atmosphere of air/CO₂ (19:1), the medium was removed by aspiration. The plates were rinsed three times and the cells were incubated for 18 h in fresh DMEM without foetal bovine serum and dexamethasone unless otherwise indicated. In experiments involving down-regulation of protein kinase C, the medium also contained TPA or other activators of this enzyme as shown in the Figure legends, and 1% dimethyl sulphoxide solvent was added to the control medium.

For glycogen synthase measurements, hepatocytes were preincubated for 15 min with 50 mM-glucose to activate the enzyme before being incubated for a further 15 min with hormones or other agents. Glycogen synthase activity was assayed by the method of Thomas *et al.* (1968), with incubation at 37 °C for 30 min. Glycogen phosphorylase *a* activity was measured as previously described (Hutson *et al.*, 1976). The incorporation of ¹⁴C from 30 mM-[U-¹⁴C]glucose into glycogen was measured by the method of Chan & Exton (1976). Protein content was determined by the method of Lowry *et al.* (1951).

Radioligand-binding study

[³H]AVP (60–75 Ci/mmol) binding was performed with intact cells (250–350 μ g of protein/well) in 12-well plates. The cells were incubated in DMEM containing 0.25% bacitracin, 0.25% bovine serum albumin and 0.05–75 nM-[³H]AVP in a final volume of 500 μ l. All steps were carried out at room temperature. After incubation for 15 min, the reaction was stopped by washing the plates with 3 $\times$ 4 ml of ice-cold phosphate-buffered saline. The cells were incubated for 10 min at 37 °C in 0.5 ml of trypsin (0.25%, w/v), then harvested by scraping the hepatocytes from the plate by the use of a rubber policeman. They were then transferred to minivials containing 5 ml of Beckman Ready-Solv economic pre-mixed (EP) scintillation cocktail, and radioactivity was measured.

Non-specific binding was simultaneously determined in replicate wells in the presence of unlabelled vasopressin (10 μ M). Specific binding (65–75% of total binding) was defined as the difference between total and non-specific binding.

Protein kinase assays

Protein kinase C activity was assayed by measuring the incorporation of ³²P from [γ -³²P]ATP into histone IIIS after partial purification of the enzyme. For each determination, five dishes (60 mm diam.) (equivalent to 6–7 mg of protein) were washed three times with ice-cold phosphate-buffered saline. The cells were harvested and incubated for 25 min in 20 mM-Hepes buffer (pH 7.5) containing 5 mM-DTT, 2 mM-EDTA, 5 mM-EGTA, 0.25 M-sucrose, 1 mM-phenylmethanesulphonyl fluoride, 0.5% Triton X-100 and 100 μ g each of leupeptin and

antipain/ml. The solubilized protein kinase C was then centrifuged at 50 000 *g* for 1 h. Owing to the inability to measure protein kinase C in the crude preparations because of low activity, partial purification with DEAE-cellulose was carried out as described elsewhere (McArdle *et al.*, 1987; Navarro, 1987; Krug & Tashjian, 1987). The supernatant was applied to a 0.3 ml DEAE-Sepharose (DE52) column equilibrated with Hepes buffer (pH 7.5) containing 5 mM-DTT, 1 mM-EGTA and 0.5 mM-EDTA. The column was washed with 5 ml of the same buffer, then with 3 ml of 20 mM-Hepes buffer (pH 7.5) containing 5 mM-DTT, 1 mM-EGTA, 0.5 mM-EDTA and 20 mM-NaCl. The protein kinase C was eluted with 0.9 ml of 20 mM-Hepes buffer (pH 7.5) containing 5 mM-DTT, 0.5 mM-EDTA, 1 mM-EGTA and 300 mM-NaCl. The protein kinase C assay contained 25 mM-MgCl₂, 20 mM-Hepes, 100 μ M-ATP, [γ -³²P]ATP (5×10^6 c.p.m.), 90 μ g of histone IIIS, 30 μ g of phosphatidylserine and 1 μ g of diolein, or 2 mM-EGTA for the control. The reaction was started by adding 35 μ l of partially purified protein kinase C to give a total volume of 110 μ l. Incubations were conducted at 30 °C for 3 min and were stopped with 30 μ l of acetic acid. Samples (100 μ l) were applied to phosphocellulose P-81 paper (Whatman) and washed several times in 0.05% H₃PO₄ as described by Reskoko (1983).

Presentation of data

All measurements were performed in duplicate or triplicate and the variation usually was less than 10% of the mean. Specific [³H]AVP-binding data were analysed by the non-linear curve-fitting model using the RS-1 program on an IBM PC computer, and the Scatchard plots of the binding data were linear ($r = 0.96 \pm 0.03$). Unless otherwise indicated, each experiment was done at least three times with separate preparations of cultured cells, and the mean values are presented in the Figures.

Materials

[³H]AVP was purchased from New England Nuclear. [U-¹⁴C]Glucose 1-phosphate, UDP-[U-¹⁴C]glucose and [U-¹⁴C]glucose were from ICN. Phosphatidylserine, Hepes, ATP, AVP, histone IIIS, TPA, mezerein, bovine serum albumin (fraction V) and dexamethasone were from Sigma. DEAE-cellulose (DE52) was from Whatman. Components for tissue culture were obtained from Gibco and Falcon. Leupeptin and antipain were from Transformation Research. Foetal bovine serum was from Hyclone Laboratory.

RESULTS

Effects of vasopressin, glucagon, isoprenaline and ATP on glycogen synthesis and glycogen phosphorylase after pretreatment of cultured hepatocytes with TPA

Fig. 1 shows the effects of pretreatment of cultured hepatocytes with 1 μ M-TPA for 18 h on the ability of vasopressin (100 nM), glucagon (100 nM), isoproterenol (isoprenaline; 10 μ M) and ATP (100 μ M) to inhibit glycogen synthesis and activate glycogen phosphorylase. The TPA treatment had no significant effect on basal glycogen synthesis, but greatly decreased the inhibitory effect of vasopressin (Fig. 1a). The same effect was observed when the agonist was ATP, which also acts by mobilizing intracellular Ca²⁺ (Charest *et al.*, 1985). In

Fig. 1. Effect of TPA on the ability of AVP, glucagon, isoprenaline and ATP to activate phosphorylase and inhibit glycogen synthesis in cultured hepatocytes

After 4 h in culture in DMEM supplemented with dexamethasone (1 μ M), defined foetal bovine serum (10%) and bovine serum albumin (1%), the cells were maintained in medium supplemented with 1% bovine serum albumin only, in the presence of 1% dimethyl sulphoxide as control ($\square$) or in the presence of 1 μ M-TPA ($\boxtimes$) dissolved in dimethyl sulphoxide. After an additional 18 h incubation, the cells were washed several times and further incubated with AVP (100 nM), glucagon (GLU; 100 nM) isoprenaline (ISO; 10 μ M) or ATP (100 μ M): CTL, control. Glycogen synthesis from 30 mM-[U- 14 C]glucose (added at the same time as the hormones) (a) and phosphorylase a activity (b) were measured as described in the Experimental section at 15 and 5 min respectively. Phosphorylase a activity is expressed as units/mg of protein, and glycogen synthesis as nmol of [U- 14 C]glucose incorporated into glycogen/h per mg of protein.

Fig. 2. [3 H]AVP specific binding to intact cells after 18 h incubation in the absence or presence of 1 μ M-TPA

The hepatocytes were seeded at a concentration of 4×10^4 cells/cm 2 in 12-well plates and incubated for 4 h in DMEM with 10% foetal bovine serum, 1% bovine serum albumin and 1 μ M-dexamethasone. Half the plates were further incubated in medium supplemented with 1% bovine serum albumin and with 1 μ M-TPA ($\bullet$) or 1% dimethyl sulphoxide ($\circ$) for 18 h. [3 H]AVP binding was measured as described in the Experimental section. Non-specific binding was determined by adding 10 μ M-vasopressin to the cells. Specific binding was 60–75% of total binding. The dissociation constant, K_d , and maximum number of binding sites, B_{max} , were evaluated by a non-linear curve-fitting analysis by using the RS-1 program. Results are means $\pm$ S.E.M. of replicates from two different experiments. For controls, K_d = 4.5 nM and B_{max} = 63 fmol/mg; for TPA-treated, K_d = 4 nM and B_{max} = 66 fmol/mg.

contrast, TPA did not produce any significant modification of the effects of glucagon and isoprenaline, both of which act through adenylate cyclase activation.

As shown in Fig. 1(b), the treatment with TPA did not affect the activation of glycogen phosphorylase by any of the agents tested. For our further experiments, we selected one Ca^{2+} -mobilizing agonist, namely vasopressin, since this does not interact with adenylate cyclase. In accord with the data of Fig. 1(b), showing that phosphorylase activation by vasopressin was unaffected by preincubation of hepatocytes for 18 h with 1 μ M-TPA, Fig. 2 shows that this treatment did not change the binding of [3 H]AVP with respect to either its K_d or B_{max} , thus excluding an effect of TPA on the receptor.

Effect of TPA preincubation on glycogen synthase regulation in cultured hepatocytes

Fig. 3 shows the effects of vasopressin and glucagon on the activity ratio of glycogen synthase in cultured hepatocytes. The cells were incubated for 18 h in presence of 10% foetal bovine serum and 1 μ M-dexamethasone with or without 1 μ M-TPA. They were then incubated for 15 min with 50 mM-glucose (to activate glycogen synthase) and then for 15 min more with 100 nM-AVP or -glucagon. As noted above for glycogen synthesis, TPA treatment markedly decreased AVP inhibition of glycogen synthase activity. In the absence of TPA, AVP inhibited glycogen synthase by 35%, and TPA blocked this effect by approx. 80%. However, TPA did not significantly inhibit the effect of glucagon on glycogen synthase activity. Incubation of cultured hepatocytes for 18 h with TPA also totally reversed the inhibition of glycogen synthase induced by short-term exposure to itself (Fig. 4). In contrast, the inhibition of glycogen

Fig. 3. Effect of AVP and glucagon to inactivate glycogen synthase after 18 h initial incubation in the absence or presence of TPA

After 4 h in culture, the cells were washed and maintained in medium containing 10% foetal bovine serum and 1 μ M-dexamethasone in the presence of 1 μ M-TPA (▨) or 1% dimethyl sulphoxide (□) for 18 h. To measure glycogen synthase, the hepatocytes were preincubated for 15 min with 50 mM-glucose to activate glycogen synthase. The cells were further incubated with AVP (100 nM) or glucagon (GLU; 100 nM) for 15 min more; CTL, control. The glycogen synthase activity ratio is the activity of glycogen synthase measured in the absence of glucose 6-phosphate divided by the activity measured in the presence of 10 mM-glucose 6-phosphate. Results are means $\pm$ S.E.M. of three different experiments assayed in duplicate.

Fig. 4. Effect of TPA treatment on the ability of AVP, A23187 and TPA to inactivate glycogen synthase

For details, see the legend to Fig. 3. After the initial 4 h in culture, the cells were incubated in the presence of 1 μ M-TPA (▨) or 1% dimethyl sulphoxide (□). After 18 h, the plates were washed and further incubated for 15 min with 50 mM-glucose and then for 15 min with 100 nM-AVP, 1 μ M-A23187 or 1 μ M-TPA; CTL, control. Each value is the mean $\pm$ S.E.M. from three different experiments performed and assayed in duplicate.

synthase activity induced by the Ca^{2+} ionophore A23187 was not impaired by preincubation with TPA (Fig. 4).

Previous studies from our laboratory (Bocckino *et al.*, 1985) have shown that the ionophore A23187 increases

Fig. 5. Concentration-dependence of TPA effect to reverse glycogen synthase inhibition by AVP

After the initial 4 h in culture, the hepatocytes were further incubated for 18 h with increasing concentrations of TPA (0.1 nM–1 μ M). The control (CTL) cells were incubated with 1% dimethyl sulphoxide. [^{14}C]Glucose and AVP were added simultaneously at concentrations of 30 mM and 100 nM respectively. After 15 min, the plates were harvested and the glycogen was measured as described in the Experimental section. The results represent the mean values of triplicate determinations from four different experiments. $\text{EC}_{50} = 10$ nM.

Fig. 6. Dose-response of AVP to inhibit glycogen synthesis after preincubation in the absence or presence of TPA

See the legend of Fig. 4 for details. The cells were incubated for 18 h with 1 μ M-TPA (●) or 1% dimethyl sulphoxide (■). Each value is the mean $\pm$ S.E.M. from three different experiments performed in triplicate and assayed in duplicate. CTL, control. EC_{50} : for control, 0.4 nM; for TPA-treated, 7.5 nM.

diacylglycerol in hepatocytes, raising the possibility that it may inactivate glycogen synthase in part through a mechanism involving protein kinase C. However, Fig. 4 shows that TPA treatment did not modify the effect of the ionophore. This may be because at 1 μ M concentration it causes greater changes in cell Ca^{2+} and smaller changes in diacylglycerol than do agonists such as vasopressin (Blackmore *et al.*, 1978; Bocckino *et al.*, 1985). In other words, its actions are probably mediated mainly by Ca^{2+} .

Fig. 7. Dose-response of TPA on protein kinase C activity (a) and on AVP inactivation of glycogen synthase (b)

After the initial 4 h in culture, the cells were maintained in the presence of 1% dimethyl sulphoxide (CTL) or TPA (0.1 nM, 10 nM or 1 μM). After an additional 18 h incubation, the cells were harvested and pooled (6–7 mg of protein), and protein kinase C was assayed as described in the Experimental section. To measure glycogen synthase activity, the cells were incubated for 15 min with 50 mM-glucose and then for 15 min with 100 nM-AVP. Each point represents the mean $\pm$ S.E.M. of four different experiments.

Fig. 8. Effect of TPA, 4 α -phorbol and mezerein on vasopressin inhibition of glycogen synthesis (a), glycogen synthase (b) and protein kinase C (c) activity

After the initial 4 h in culture, the cells were maintained for 18 h more in presence of 1% dimethyl sulphoxide (CTL, AVP) or 1 μM-TPA (TPA), 4 α -phorbol (4AP) or -mezerein (MEZ). AVP (100 nM) was then added to all plates except control (CTL) and those used to assay protein kinase C. Glycogen synthase and glycogen synthesis were assayed as described in the Experimental section. Each value for glycogen synthesis and glycogen synthase (measured as described in the legends to Figs. 1 and 3) is the mean $\pm$ S.E.M. of triplicate determinations assayed in duplicate. The protein kinase C data are representative of two different experiments.

Concentration-dependence of the effect of TPA to reverse the inhibition of glycogen synthesis by vasopressin

Cultured hepatocytes were incubated for 18 h with increasing concentrations of TPA (0.1 nM–1 μM) to determine the dose-response curve to reverse the inactivation of glycogen synthesis induced by 100 nM-vasopressin. The control rate of glycogen synthesis, in nmol of [14 C]glucose incorporated/h per mg of protein was 43.5 ± 1.5 (measured in the absence of TPA or vasopressin), and maximally effective concentrations of

TPA (0.1–1 μM) reversed the vasopressin effect, from 27.5 ± 0.8 to 36.3 ± 0.6 . Half-maximal inhibition was obtained with 10 ± 2 nM-TPA (Fig. 5).

We also preincubated the hepatocytes in the presence or absence of 1 μM-TPA and measured glycogen synthesis in the presence of different concentrations of vasopressin (Fig. 6). Maximum inhibition of glycogen synthesis ($32 \pm 5\%$) was observed with 10–100 nM-vasopressin. As expected, TPA (1 μM) decreased the magnitude of the inhibition by approx. 50%. There was also a shift in the EC_{50} for vasopressin, from 0.4 ± 0.2 to 7.5 ± 0.6 nM.

Effects of different concentrations of TPA and different tumour promoters on protein kinase C activity and on vasopressin inhibition of glycogen synthase and glycogen synthesis

In view of the fact that TPA treatment did not completely inhibit the action of vasopressin on glycogen synthase or synthesis, further studies were carried out to determine the magnitude of down-regulation of protein kinase C under the experimental conditions used in the above studies.

Fig. 7(a) shows that incubation of cultured hepatocytes with 1 μ M-TPA led to a dramatic inactivation of protein kinase C (Fig. 7a), from 11.2 ± 2.3 to 1.5 ± 1.1 units/mg of protein. Partial inactivation was observed with 10 nM-TPA, and essentially no inhibition with 0.1 nM. Furthermore, there was an excellent correlation ($r = 0.98$) between protein kinase C inactivation and loss of the ability of vasopressin to inactivate glycogen synthase (Fig. 7b).

To establish selectivity, another activator of protein kinase C and an inactive phorbol were tested. Mezerein, a non-phorbol-ester diterpene, has been classified as a tumour promoter (Miyake *et al.*, 1984; Furstenberger *et al.*, 1981). It can also displace 4 β -phorbol 12,13-dibutyrate from its binding site (Goodwin & Weinberg, 1982). Mezerein (1 μ M) produced almost the same down-regulation of protein kinase C and reversal of the effect of vasopressin on glycogen synthase and synthase as did 1 μ M-TPA (Fig. 8). However, when 4 α -phorbol, an inactive phorbol, was used, it did not produce any effect on either glycogen synthase inactivation or protein kinase C activity. In these studies, in which the same cell preparations were used, there were good correlations between the effects of the agents on glycogen synthase and synthesis.

DISCUSSION

The results of the present study demonstrate a role for protein kinase C in the regulation of hepatic glycogen synthase by Ca^{2+} -mobilizing agonists. Early studies hypothesized that glycogen synthase was inactivated through direct and indirect pathways, involving an increase in Ca^{2+} and activation of phosphorylase *b* kinase (Strickland *et al.*, 1980, 1983), phosphorylation of glycogen synthase by Ca^{2+} -calmodulin-dependent protein kinase (Payne & Soderling, 1980; Payne *et al.*, 1983) and inhibition of glycogen synthase phosphatase by Ca^{2+} (Van de Werve, 1981; Mvumbi *et al.*, 1985). However, Ca^{2+} -mobilizing agonists, such as vasopressin, angiotensin II, P_2 -purinergic and α_1 -adrenergic agonists, induce not only the accumulation of inositol 1,4,5-trisphosphate, with release of Ca^{2+} , but also the accumulation of diacylglycerol (Bocckino *et al.*, 1985; Bouscarel & Exton, 1986), which activates protein kinase C (Nishizuka, 1984; Ashendel, 1985). This enzyme has been shown to be involved in many biological effects, and may regulate glycogen synthase. Roach & Goldman (1983) first showed that phorbol ester added to isolated hepatocytes inactivated glycogen synthase, but did not alter phosphorylase activity. Blackmore *et al.* (1986) also observed that the inactivation of glycogen synthase by TPA or vasopressin was still present in Ca^{2+} -depleted cells, and concluded that a mechanism existed for the inactivation of glycogen synthase in liver cells that was

independent of increases in cell Ca^{2+} and involved protein kinase C. This idea was reinforced by the studies by Bouscarel & Exton (1986), which showed good correlations between changes in diacylglycerol and glycogen synthase in hepatocytes incubated with angiotensin II or noradrenaline.

On the other hand, studies *in vitro* of the phosphorylation of liver glycogen synthase by protein kinase C (Imazu *et al.*, 1984; Nakabayashi *et al.*, 1987) showed that, although the enzyme was significantly phosphorylated (0.5–1.4 mol of phosphate/mol of subunit), there was no detectable inactivation of the enzyme, in contrast with a previous report, where muscle glycogen synthase was the substrate (Ahmad *et al.*, 1984).

Treatment of cells with tumour-promoting phorbol esters not only activates and causes rapid translocation of the enzyme from the cytosol to the membrane, but can cause marked down-regulation of the enzyme (Darbon *et al.*, 1986; Rodriguez-Pena & Rozengurt, 1984). In agreement with results in many cell types, incubation of hepatocytes in primary culture with TPA for 18 h led to a complete loss of detectable protein kinase C activity (Figs. 7 and 8c). The effect was time-dependent (results not shown), and the extent of inhibition of the enzyme was dependent on the TPA concentration (Fig. 7). It was also specific for activators of the enzyme (Fig. 8c). For example, it was not seen with 4 α -phorbol, which does not displace [^3H]4 β -phorbol 12,13-dibutyrate from a binding site on murine fibroblastic cells (Collins & Rozengurt, 1982) or produce thyroid-cell proliferation, unlike TPA (Roger *et al.*, 1986). On the other hand, mezerein, a non-phorbol tumour promoter from *Daphne mezereum*, produced the same effects as TPA (Fig. 8). Several authors have reported that mezerein acts by a TPA-like mechanism (O'Flaherty *et al.*, 1985; Miyake *et al.*, 1984).

Under conditions where TPA completely down-regulated protein kinase C, it only partially blocked the inhibition of glycogen synthase (Figs. 3, 4, 7b and 8b) or glycogen synthesis (Figs. 1a, 5, 6, 8a) by vasopressin. The incomplete inhibition of the vasopressin effects indicates that other mechanisms are involved which are not dependent on protein kinase C. These presumably involve phosphorylase *b* kinase, glycogen synthase phosphatase or Ca^{2+} -calmodulin-dependent protein kinase, and may be as important as those involving protein kinase C.

The existence of protein kinase C-independent mechanisms involving Ca^{2+} is also shown by the fact that TPA preincubation did not significantly change the inhibitory effect of the Ca^{2+} ionophore A23187 (Fig. 4). Despite the apparent existence in liver cells of both Ca^{2+} -dependent and protein kinase C-dependent mechanisms for glycogen synthase inactivation, there is no evidence that they are synergistic (Blackmore *et al.*, 1986), in contrast with what is seen in some systems (Nishizuka, 1984).

TPA treatment inhibits the receptor binding of α_1 -adrenergic agonists, muscarinic cholinergic agonists and epidermal growth factor in several cell lines (for reference see Corvera *et al.*, 1986; Serra *et al.*, 1986; Davis *et al.*, 1985). However, it did not decrease the affinity or number of binding sites for vasopressin in cultured hepatocytes (Fig. 2). Its major action was to down-regulate protein kinase C. This is thought to occur by translocation of the enzyme to the membrane (Kraft & Anderson, 1983; Ballester & Rosen, 1985), followed by a partial proteolysis as described in breast-cancer cells

(Fabbro *et al.*, 1986; Darbon *et al.*, 1986; Chida *et al.*, 1986).

A major unresolved problem raised by the present and other studies relates to the mechanism by which protein kinase C activation leads to inactivation of glycogen synthase in liver cells. As noted above, studies of the direct phosphorylation of hepatic glycogen synthase by protein kinase C have revealed no inactivation (Imazu *et al.*, 1984; Nakabayashi *et al.*, 1987). It is conceivable that inactivation could be observed under different assay conditions, or that a factor analogous to the activator protein of tryptophan hydroxylase (Yamauchi *et al.*, 1981) is required in order to see the effects of phosphorylation. Alternatively, the effect of protein kinase C could be indirect, i.e. through activation of another glycogen synthase kinase or inhibition of a protein phosphatase. Finally, prior or simultaneous phosphorylation of the synthase by another kinase may be necessary in order to see activity changes with protein kinase C.

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